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Metabolic profile of wound-induced changes in primary carbon metabolism in sugarbeet root

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ABSTRACT

Injury to plant products by harvest and postharvest operations induces respiration rate and increases the demand for respiratory substrates. Alterations in primary carbon metabolism are likely to support the elevated demand for respiratory substrates, although the nature of these alterations is unknown. To gain insight into the metabolic changes that occur to provide substrates for wound-induced increases in respiration, changes in the concentrations of compounds that are substrates, intermediates or cofactors in the respiratory pathway were determined in sugarbeet (*Beta vulgaris* L.) roots in the 4 days following injury. Both wounded and unwounded tissues of wounded roots were analyzed to provide information about localized and systemic changes that occur after wounding. In wounded tissue, respiration increased an average of 186%, fructose, glucose 6-phosphate, ADP and UDP concentrations increased, and fructose 1,6-bisphosphate, triose phosphate, citrate, isocitrate, succinate, ATP, UTP and NAD^+ concentrations decreased. In the non-wounded tissue of wounded roots, respiration rate increased an average of 21%, glucose 6-phosphate, fructose 6-phosphate, glucose 1-phosphate and ADP concentrations increased, and isocitrate, UTP, NAD^+, $NADP^+$, and NADPH concentrations declined. Changes in respiration rate and metabolite concentrations indicated that localized and systemic changes in primary carbon metabolism occurred in response to injury. In wounded tissue, metabolite concentration changes suggested that activities of the early glycolytic enzymes, fructokinase, phosphofructokinase, phosphoglucose isomerase, and phosphoglucomutase were limiting carbon flow through glycolysis. These restrictions in the respiratory pathway, however, were likely overcome by use of metabolic bypasses that allowed carbon compounds to enter the pathway at glycolytic and tricarboxylic acid (TCA) cycle downstream locations. In non-wounded tissue of wounded roots, metabolic concentration changes suggested that glycolysis and the TCA cycle were generally capable of supporting the small systemic elevation in respiration rate. Although the mechanism by which respiration is regulated in wounded sugarbeet roots is unknown, localized and systemic elevations in respiration were positively associated with one or more indicators of cellular redox status.

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1. Introduction

Plant products inevitably are wounded from harvest, transport and storage operations. Wounding triggers an array of responses including cell division and the biosynthesis of callose, suberin, lignin, phytoalexins, and structural and defense proteins to assist in sealing the wound site from the environment, repairing damaged tissue, minimizing dehydration, and defending against opportunistic pathogens (reviewed in de Bruxelles and Roberts, 2001; Léon et al., 2001). Wounding also induces respiration, presumably to provide energy for these anabolic processes (Lipetz, 1970). Increases in respiration due to mechanical injury have been documented in numerous harvested plant products and are typically large, with respiration rate commonly increasing several fold (Passam et al., 1976; Kays and Paull, 2004; Serrano et al., 2004).

The increase in respiration in response to wounding is likely to require alterations in plant primary carbon metabolism. Although amino acids, proteins, lipids and organic acids can serve as substrates for respiration, carbohydrates, especially sucrose and starch, are the most common substrates for the process (Siedow and Day, 2000). Respiration of carbohydrates requires activity of sucrose and/or starch degrading enzymes as well as operation of the glycolytic pathway and the tricarboxylic acid (TCA) cycle. Combined these pathways provide the reduced compounds needed to fuel the electron transport chain and catalyze ATP formation via oxidative phosphorylation. While the flow of carbon through primary carbon catabolic pathways must increase to provide for the increased use of respiratory substrates in wounded plant organs, it is unknown how plants alter their metabolism to achieve this.

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Determining the mechanism(s) by which metabolism is altered to support the large increases in respiration caused by wounding is complicated by the complexity of the respiratory pathway (Fig. 1). Sucrose degradation can occur by the action of three different enzyme activities, starch degradation requires at least three enzyme activities, and glycolysis and the TCA cycle require at least 10 and 9

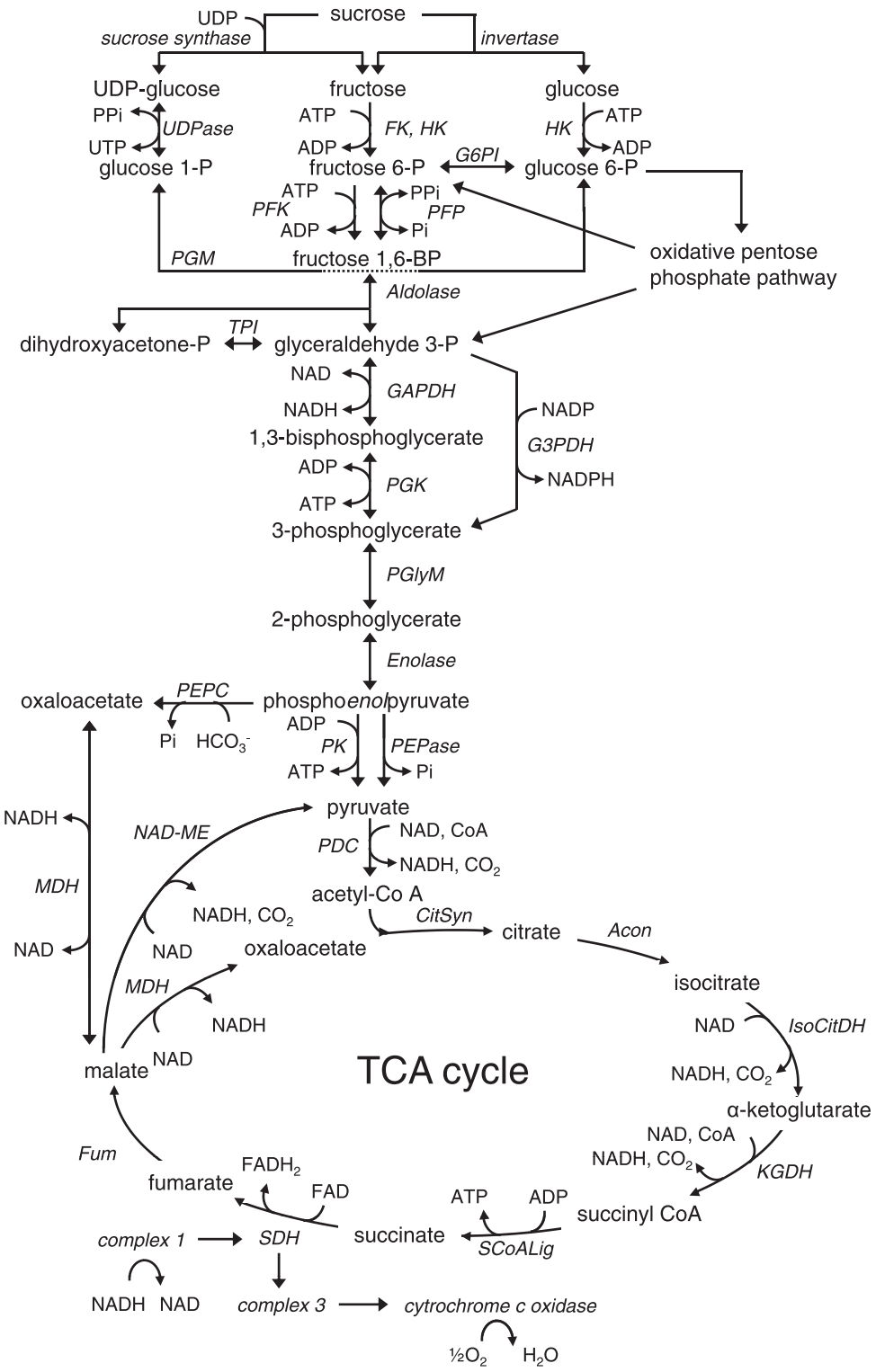

Fig. 1. Schematic of the enzymatic steps and metabolic intermediates in the respiration of sucrose. Enzyme abbreviations: Acon, aconitase; CitSyn, citrate synthase; FK, fructokinase; Fum, fumarase; G3PDH, NADP-dependent non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase; G6PI, glucose 6-phosphate isomerase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HK, hexokinase; IsoCitDH, isocitrate dehydrogenase; KGDH, α-ketoglutarate dehydrogenase; MDH, malate dehydrogenase; NAD-ME, NAD-malic enzyme; PDC, pyruvate dehydrogenase complex; PEPase, phospho*enol*pyruvate phosphatase; PEPC, phospho*enol*pyruvate carboxylase; PFK, phosphofructokinase; PFP, pyrophosphate-dependent phosphofructokinase; PGK, phosphoglycerate kinase; PGlyM, phosphoglyceromutase; PGM, phosphoglucomutase; PK, pyruvate kinase; SCoALig, succinyl coenzyme A ligase; SDH, succinate dehydrogenase; TPI, triose phosphate isomerase; UDPase, UDP-glucose pyrophosphorylase. Chemical compound abbreviations: CoA, coenzyme A; dihydroxyacetone-P, dihydroxyacetone phosphate; fructose 1,6-BP, fructose 1,6-bisphosphate; fructose 6-P, fructose 6-phosphate; glucose 1-P, glucose 1-phosphate; glucose 6-P, glucose 6-phosphate; glyceraldehyde 3-P, glyceraldehyde 3-phosphate; Pi, inorganic phosphate; PPi, inorganic pyrophosphate.

different enzyme activities, respectively, for their operation. In addition, several enzymatic steps in these pathways can be performed by either alternative enzymes or bypassed by other metabolic pathways such as the oxidative pentose phosphate pathway, the glyoxylate cycle or a phospho*enol*pyruvate carboxylase/malate dehydrogenase bypass of glycolytic end-product reactions.

Metabolic profiling has proven to be a useful tool in evaluating complex alterations in metabolism (Stitt et al., 2010). Metabolic profiles have been used to examine global changes in metabolism or targeted for the analysis of metabolic changes specific to one or more pathways (Shulaev et al., 2008). The technique has been employed in a number of plant species and successfully used to determine relationships between metabolites, identify biomarkers related to biological traits or stress conditions, and provide insights into the regulation of metabolic pathways (Roessner et al., 2001; Stitt and Fernie, 2003; Shulaev et al., 2008).

In research described here, metabolic profiling was used to provide insight into the alterations that occur in primary carbon metabolism to support wound-induced increases in respiration. Concentrations of compounds that are substrates, intermediates, or cofactors of the respiratory pathway were quantified in wounded sugarbeet (*Beta vulgaris* L.) roots in the 4 days following injury and compared to those found in comparable tissue from unwounded roots. Metabolic changes were determined in the wounded and unwounded tissues of wounded roots to provide information about localized as well as systemic changes in primary carbon metabolism that occur in response to injury. Sugarbeet roots were used for the study's experimental material since wound-induced respiration is an economically important problem for the sugarbeet industry and they provide a simple system to study primary carbon metabolism. Sugarbeet roots sustain significant damage due to harvest and storage operations, and large increases in respiration rate and storage sucrose losses due to wounding have been documented (Wyse, 1978; Steensen, 1996). Sugarbeet roots also provide an ideal system to study primary carbon metabolism, since their respiration is fueled almost entirely by sucrose, is uncomplicated by starch catabolism or the use of multiple respiratory substrates, and proceeds by a linear progression of sucrolysis, glycolysis and the TCA cycle with limited participation by the oxidative pentose phosphate cycle (Barbour and Wang, 1961; Wang and Barbour, 1961).

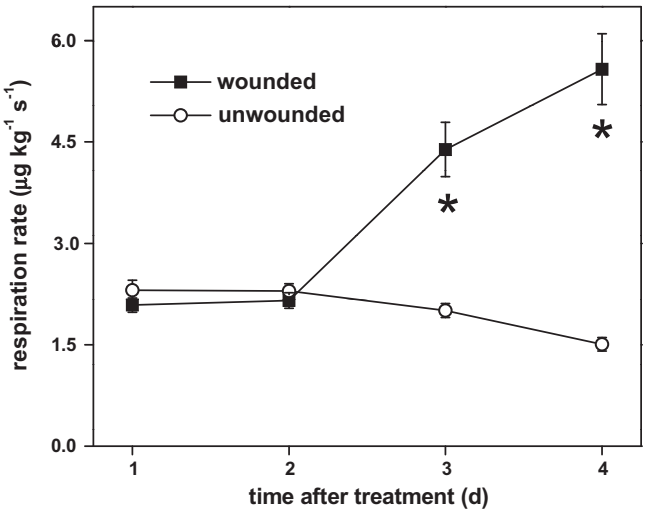

Fig. 2. Respiration rate of wounded and unwounded sugarbeet roots during storage at 10 °C for 4 days. Root respiration was measured as CO_2 production. Statistically significant treatment × day interactions are identified with asterisks. Data are the mean ± SE of the mean (n = 4).

2. Results and discussion

2.1. Wound effects on root respiration

Wounding increased the respiration rate of sugarbeet roots relative to that of unwounded roots beginning on the third day after injury (Fig. 2). Respiration rates for wounded and unwounded roots stored at 10 °C were similar in the first 2 days after injury. Three days after injury, wounded roots respired at a rate that was 120% greater than that of unwounded roots. Four days after injury, the respiration rate of wounded roots was elevated by 270%.

Wounding increased respiration throughout the root even though injury was evident only in the root's surface and peripheral tissues (Fig. 3). Wound effects on respiration, however, were greatest in the peripheral tissues that sustained injury. In these tissues, respiration was elevated an average of 186% above that of similar tissue from unwounded roots for the 4-day duration of the experiment, with the greatest increase occurring 3 and 4 days after

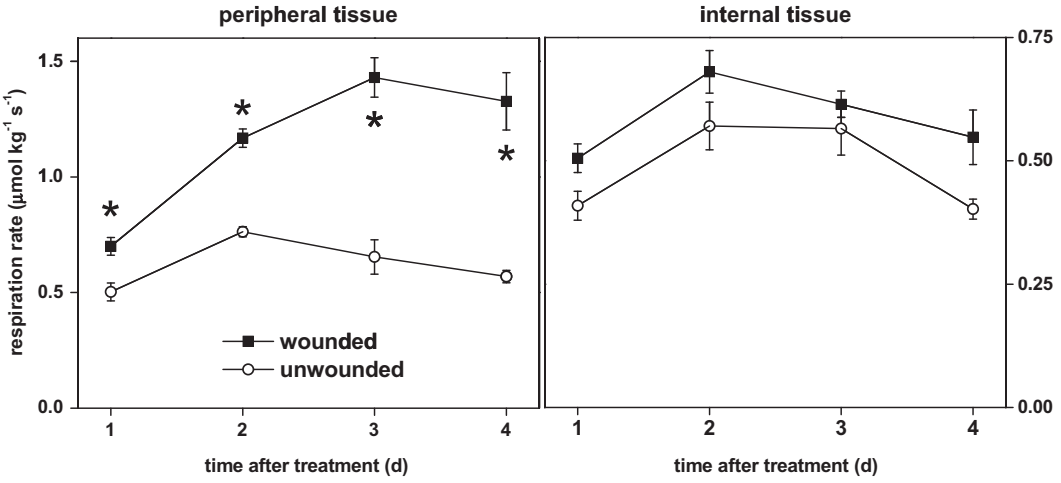

Fig. 3. Respiration rate of peripheral and internal tissues of wounded and unwounded sugarbeet roots during storage at 10 °C for 4 days. Peripheral tissue contained the visibly damaged tissue from wounded roots and comparable tissue from unwounded roots; internal tissue contained tissue that did not sustain any visible damage. Respiration was measured as O_2 consumption. Statistically significant treatment x day interactions are identified with asterisks. Data are the mean ± SE of the mean (n = 4). Scale of the y axis differs between panels.

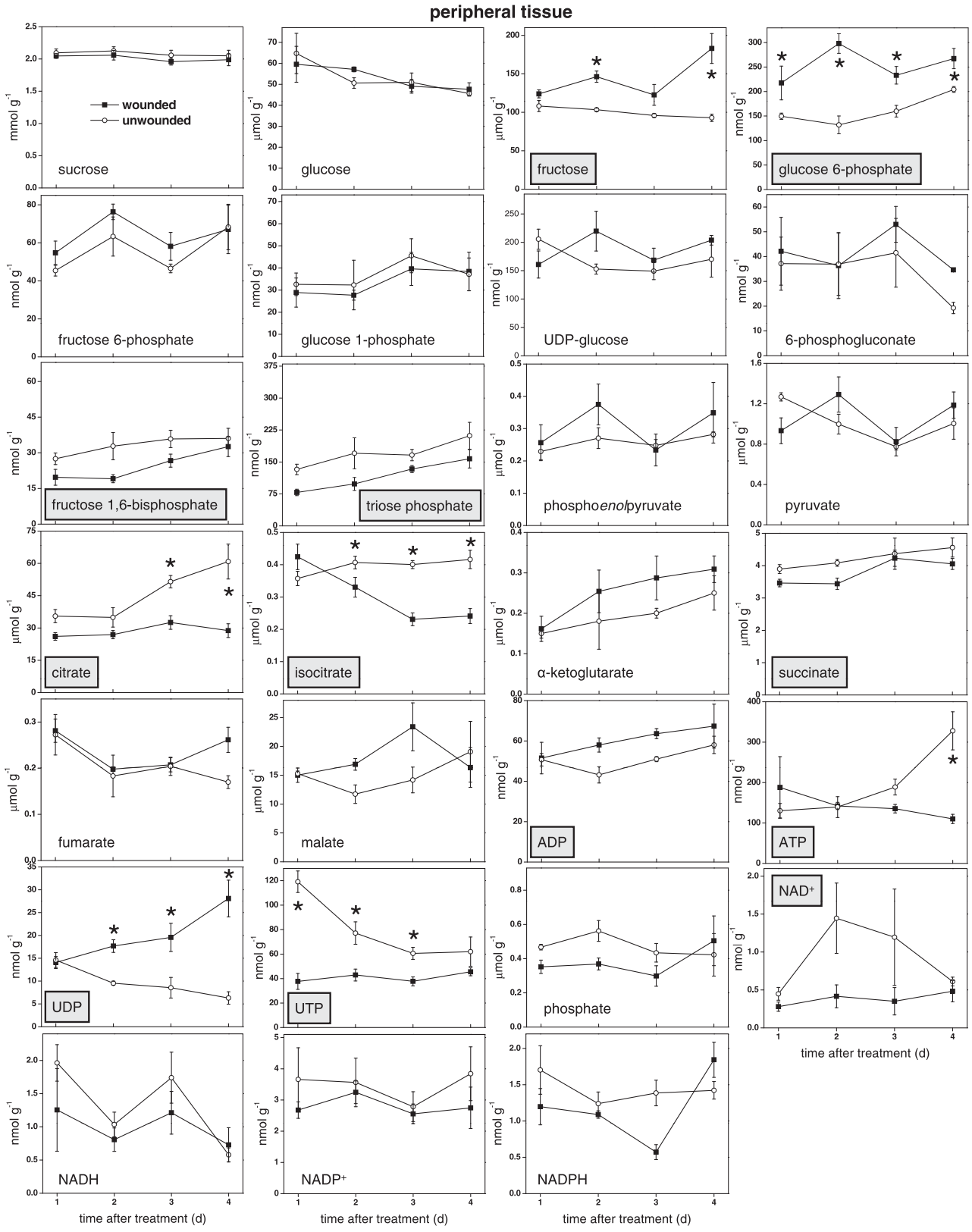

Fig. 4. Concentrations of substrates and intermediates in the respiratory pathway in peripheral tissues of wounded and unwounded roots during storage at 10 °C for 4 days as a function of time. Peripheral tissue contained the visibly damaged tissue from wounded roots and comparable tissue from unwounded roots. Concentrations were determined on a dry weight basis. Metabolites that are statistically different ($\alpha = 0.05$) between wounding treatments are highlighted by a shaded box surrounding the metabolite name. Statistically significant treatment × day interactions are identified with asterisks. Data are the mean ± SE of the mean ($n = 4$).

injury. Respiration also significantly increased in the internal tissues of wounded roots, although these tissues sustained no visual damage. On average, internal tissues of wounded roots respired at a rate that was 21% greater than that of similar tissue from unwounded roots, with the greatest elevation in respiration occurring on the fourth day after injury.

Induction of respiration in response to injury occurs in nearly all plant organs (Kays and Paull, 2004). In sugarbeet roots, wound induction of respiration is well documented and is believed to occur to provide substrates and metabolic energy for wound-healing processes (Wyse and Peterson, 1979; Klotz et al., 2008). Although increases in respiration are known to occur in wounded tissues and wounded plant organs, this is, to our knowledge, the first report of systemic induction of respiration in non-wounded tissue by injury. Systemic induction of respiration is perhaps not surprising, however, since systemic changes in gene expression, protein expression and production of secondary metabolites in response to injury are typical in injured plant organs (de Bruxelles and Roberts, 2001).

2.2. Concentration changes of respiratory pathway substrates and intermediates in wounded tissue

In tissues that sustained injury, concentration changes were noted for 12 metabolites that are substrates, intermediates or cofactors in glycolysis or the TCA cycle (Fig. 4). In wounded tissue, fructose, glucose 6-phosphate, ADP and UDP concentrations significantly increased while fructose 1,6-bisphosphate, triose phosphate, citrate, isocitrate, succinate, ATP, UTP and NAD^+ concentrations significantly declined. Wounding had no statistically significant effect on the concentrations of sucrose, glucose, fructose 6-phosphate, glucose 1-phosphate, UDP-glucose, 6-phosphogluconate, phospho*enol*pyruvate, pyruvate, α-ketoglutarate, fumarate, malate, phosphate, NADH, $NADP^+$, and NADPH. Abbreviations used to describe metabolites are compiled in Table 1.

Of the soluble carbohydrates, sucrose, glucose and fructose that serve as substrates for respiration in sugarbeet roots and a carbon source for glycolysis in all plants, only fructose concentrations were significantly altered by wounding. Fructose concentrations were elevated in wounded roots throughout the 4-day duration of the experiment, with the greatest elevation occurring 4 days after injury. Accumulation of fructose indicates fructose biosynthesis via sucrolytic enzymes exceeded fructose utilization, most likely by fructokinase (FK), and suggests that FK may restrict fructose utilization in wounded tissue. Previously, FK was demonstrated to be present in sugarbeet roots at low activities (Sakalo and Tyltu, 1997; Klotz et al., 2006). Although sucrose is the principal substrate for fructose formation and respiration in sugarbeet root (Barbour and Wang, 1961; Wyse and Dexter, 1971), sucrose concentrations were not significantly reduced in wounded roots. Detecting statistically significant changes in sucrose concentration in sugarbeet root, however, is difficult since the quantity of sucrose catabolized is typically small in relation to the quantity of sucrose present (Klotz and Finger, 2004).

Table 1
Abbreviations used to describe metabolites.

Metabolite	Abbreviation
Sucrose	SUC
Glucose	GLU
Fructose	FRU
Glucose 6-phosphate	G6P
Fructose 6-phosphate	F6P
Glucose 1-phosphate	G1P
Uridine diphosphate glucose	UDPG
6-Phosphogluconate	6PGLU
Fructose 1,6-bisphosphate	FBP
Triose phosphate	TRIOP
Phospho*enol*pyruvate	PEP
Pyruvate	PYR
Citrate	CIT
Isocitrate	ISOC
α-Ketoglutarate	KETOG
Succinate	SUCC
Fumarate	FUM
Malate	MAL
Inorganic phosphate	PHOS

The significant increase in glucose 6-phosphate (G6P) concentrations and unchanging glucose concentrations suggest that hexokinase was not restricting the flux of carbon in wounded tissue. G6P concentrations in wounded root tissue were elevated an average of 57% over unwounded tissue with the increase occurring throughout the 4-day duration of the experiment. G6P concentrations were elevated in wounded tissue even though no significant changes in fructose 6-phosphate (F6P) and glucose 1-phosphate (G1P) concentrations were observed. Although hexose monophosphates are thought to be readily interconverted via near-equilibrium reversible reactions (Dennis and Blakeley, 2000), these compounds did not equilibrate in wounded sugarbeet tissue, suggesting that phosphoglucose isomerase and phosphoglucomutase, the two enzymes responsible for these interconversions, were limiting. Deviations from equilibrium distribution for hexose monophosphates have previously been noted in tissues with high metabolic activities and attributed to restrictions in phosphoglucose isomerase and phosphoglucomutase activities (Dietz, 1985; Neuhaus and Stitt, 1990). In wounded sugarbeet tissue, metabolic activity was likely to be high due to the large increase in respiration rate (Fig. 3) and the array of repair and defense mechanisms engaged by injury (Lipetz, 1970; Ibrahim et al., 2001).

Fructose 1,6-bisphosphate (FBP) and triose phosphate (TRIOP) exhibited similar declines in concentration in the peripheral tissue of wounded roots relative to unwounded tissue. Both metabolites were reduced in wounded root tissue throughout the 4-day incubation period with FBP and TRIOP concentrations declining an average of 26% and 31%, respectively, relative to unwounded peripheral tissue. FBP and TRIOP are sequential intermediates in the glycolytic pathway and are interconverted by the reversible enzyme, aldolase. Similarities in the effect of wounding and incubation time on FBP and TRIOP concentrations suggests that the two compounds were in equilibrium with each other and that sufficient aldolase activity was present to allow the metabolites to equilibrate in sugarbeet root tissue. The reduction in FBP and TRIOP concentrations in response to injury indicates that the two metabolites were utilized more rapidly than they were synthesized, possibly due to limited FBP formation by the enzyme, phosphofructokinase (PFK). Although conversion of fructose 6-phosphate to FBP can be carried out by PFK or pyrophosphate-dependent phosphofructokinase, PFK is responsible for catalysis of this reaction in most plant tissues (Dennis and Blakeley, 2000). PFK is generally believed to have a central role in restricting carbon flux through glycolysis (Dennis et al., 1997) and its ability to affect concentrations of upstream and downstream glycolytic intermediates by alterations in its activity has been demonstrated (Sweetlove et al., 2001). In sugarbeet root, PFK is present at low activities (Klotz et al., 2006).

Despite reduced concentrations of FBP and TRIOP, concentrations of the final two intermediates of the glycolytic pathway, phospho*enol*pyruvate (PEP) and pyruvate (PYR), were not affected by wounding, suggesting that carbon compounds entered glycolysis downstream of triose phosphate. Without the introduction of additional glycolytic intermediates, the reduced concentrations of FBP and TRIOP observed in wounded tissue and the reversibility of reactions catalyzing the glycolytic reactions intermediate of TRIOP and PEP would be expected to cause PEP and PYR concentrations to decline (Dennis et al., 1997). In non-photosynthetic tissue, entry of carbon into the glycolytic pathway downstream of triose phosphate is likely to occur via the activity of phospho*enol*pyruvate carboxykinase (PEPCK), an enzyme that irreversibly converts oxaloacetate to PEP. Although PEPCK activity is often associated with germination in oil seed plants or carbon fixation in the leaves

of CAM and C_4 plants, the enzyme has been found in a variety of carbohydrate-storing organs where its function is unknown (Cooper and Benedict, 1968; Blanke et al., 1988; Bahrami et al., 2001).

Carbon compounds can also be introduced into the glycolytic pathway via the oxidative pentose phosphate pathway (OPPP). Metabolite concentrations observed in this study, however, suggest that this did not occur to any great extent in wounded sugarbeet root tissue. In wounded root tissue, G6P, the substrate for the OPPP, accumulated, while concentrations of OPPP products, i.e., fructose 6-phosphate, triose phosphate and NADPH, were unaltered or reduced. In addition, concentrations of 6-phosphogluconate, the first committed intermediate of the OPPP and the product of the reaction generally considered to be the principal regulatory step of the pathway (Lendzian, 1980) were unaltered by wounding. These observations agree with findings from earlier studies that indicated that the OPPP had little contribution to carbohydrate metabolism in sugarbeet root and did not engage under stress (Wang and Barbour, 1961; Sakalo and Tyltu, 1997).

Significant reductions in the concentrations of the TCA cycle intermediates, citrate (CIT) and isocitrate (ISOC), were observed in wounded root tissue. CIT concentrations were reduced 27% in wounded tissue relative to unwounded tissue 1 day after injury, and remained at this reduced concentration for the remaining 3 days of the experiment. ISOC concentrations declined during the first 3 days after injury and were an average of 22% lower than those found in unwounded tissue. CIT and ISOC utilization, therefore, exceeded biosynthesis 1 and 1–3 days after injury, respectively. While CIT and ISOC were likely to have been used in the TCA cycle and may have become depleted by its operation, reductions in their concentrations could also have occurred by engagement of the glyoxylate cycle, a metabolic pathway in which isocitrate and acetyl-CoA, a product of fatty acid degradation, are used to produce succinate and malate. It is unknown whether the glyoxylate cycle was engaged in wounded sugarbeet tissue, although the cycle is known to be induced in senescing tissues, including those of a *B. vulgaris* subspecies (De Bellis et al., 1990; Cots et al., 2002). If engaged, the glyoxylate cycle would provide a mechanism for reassimilating carbon from damaged membranes and regenerating TCA cycle intermediates.

TCA cycle intermediates downstream of citrate and isocitrate were unaltered or minimally altered in concentration by wounding. Concentrations of α-ketoglutarate, fumarate and malate in the peripheral tissue of wounded roots did not significantly differ from concentrations found in peripheral tissue of unwounded roots. Succinate concentrations were significantly altered by wounding, but were only reduced an average of 10% in wounded tissues relative to unwounded tissue. Reductions in citrate and isocitrate concentrations, therefore, did not influence the concentrations of downstream TCA cycle enzymes. This could occur if subcellular compartmentalization of CIT and ISOC allowed cellular concentrations to be reduced without a reduction in their mitochondrial concentrations, if CIT and ISOC were present in excess such that reductions in their concentrations had no effect on concentrations of downstream TCA cycle intermediates, or if new substrates were introduced into the cycle from other pathways. Carbon compounds can enter the TCA cycle as α-ketoglutarate, succinate, and malate from amino acid catabolism, the glyoxylate cycle, the γ-aminobutyrate (GABA) shunt or a phospho*enol*pyruvate carboxylase-catalyzed bypass of the final reaction of glycolysis.

Concentrations of the TCA cycle cofactor, NAD^+, were reduced in wounded root tissue, while concentrations of other pyridine nucleotides were unaffected by injury. NAD^+ concentrations were reduced an average of 59% in the peripheral tissue of wounded roots relative to similar tissue in unwounded roots in the 4 days after injury. NADH, $NADP^+$, and NADPH concentrations were unaltered in wounded tissue. The ratios of reduced to oxidized pyridine nucleotides, i.e., the ratios of NADH:NAD^+, NADPH:$NADP^+$, and NAD(P)H:$NAD(P)^+$ were also not significantly affected by wounding (data not shown). The cause of reduced NAD^+ concentration is unknown. Reductions in NAD contents, however, typically occur due to elevated activity of poly(ADP-ribose) polymerase (PARP), a wound-inducible enzyme that uses NAD^+ as a substrate to add ADP-ribose polymers onto nuclear proteins (Amor et al., 1998; De Block et al., 2005).

Substrates for the electron transport chain were unaltered or minimally altered in concentration in wounded roots, suggesting that synthesis of respiratory substrates was nearly equal to the demand for respiratory substrates. Although respiration rate increased an average of 186% in wounded tissue (Fig. 3), NADH concentrations were not significantly altered, succinate concentrations were reduced only marginally as noted above, and NADPH, which can also be utilized as a substrate for the electron transport chain, was unaltered in concentration. The lack of any major decline in respiratory substrates suggests that metabolism was sufficiently altered in wounded roots to provide for the large demand for respiratory substrates created by injury.

Changes in ADP and ATP concentrations in wounded tissue, however, indicate that oxidative phosphorylation failed to keep pace with ATP utilization in injured tissue. ATP concentrations decreased incrementally with time after injury and declined 41% between 1 and 4 days after injury. ADP concentrations increased incrementally with time after injury and increased 31% between 1 and 4 days after injury. While ATP and ADP are, respectively, substrates and products for the early glycolytic enzymes, fructokinase, hexokinase and phosphofructokinase, changes in their concentrations may be due to metabolism via multiple enzymes, pathways and transport processes (Dennis et al., 1997).

Wounding caused significant changes in UDP and UTP concentrations in sugarbeet roots. UDP concentrations in wounded tissues doubled between 1 and 4 days after injury and were an average of 104% greater than in unwounded tissues. UTP concentrations declined an average of 51% relative to concentrations in unwounded tissues. UDP-glucose, the major uridine nucleotide-containing metabolite in sugarbeet roots, however, was unaltered by wounding. The cause of the UDP and UTP concentration changes in wounded root tissue is unknown, but may reflect callose and cell wall biosynthesis, use of UTP as a phosphate donor in enzymatic reactions, or enzymatic interconversion of adenine and uridine nucleotides. Callose and cellulose are commonly produced after injury to seal off wound sites (Stone and Clarke, 1992; Ibrahim et al., 2001). Synthesized from UDP-glucose, production of these polymers releases UDP as a reaction byproduct and could reduce UTP concentrations if UDP-glucose pyrophosphorylase, which catalyzes the reaction of UTP and glucose 1-phosphate to form UDP-glucose, participates in the production of substrate for their synthesis. Alternatively, changes in UDP and UTP concentrations could occur by the use of UTP as a phosphate source in the phosphorylation of fructose by fructokinase. While ATP is the usual phosphoryl donor for this reaction, UTP-specific fructokinases are known (Doehlert, 1990). Alterations in UDP and UTP concentrations may also arise from equilibration of uridine and adenine nucleotides such that changes in ADP and ATP concentrations cause similar changes in UDP and UTP concentrations (Dancer et al., 1990). The transfer of energy charge between nucleotide pools is catalyzed by nucleoside 5′-diphosphate kinase, an enzyme that is induced by mechanical injury in some plant organs (Harris et al., 1994).

2.3. Concentration changes of respiratory pathway substrates and intermediates in internal tissues of wounded roots

In the internal tissues of sugarbeet roots, the concentrations of 13 metabolites that are intermediates or cofactors in glycolysis or

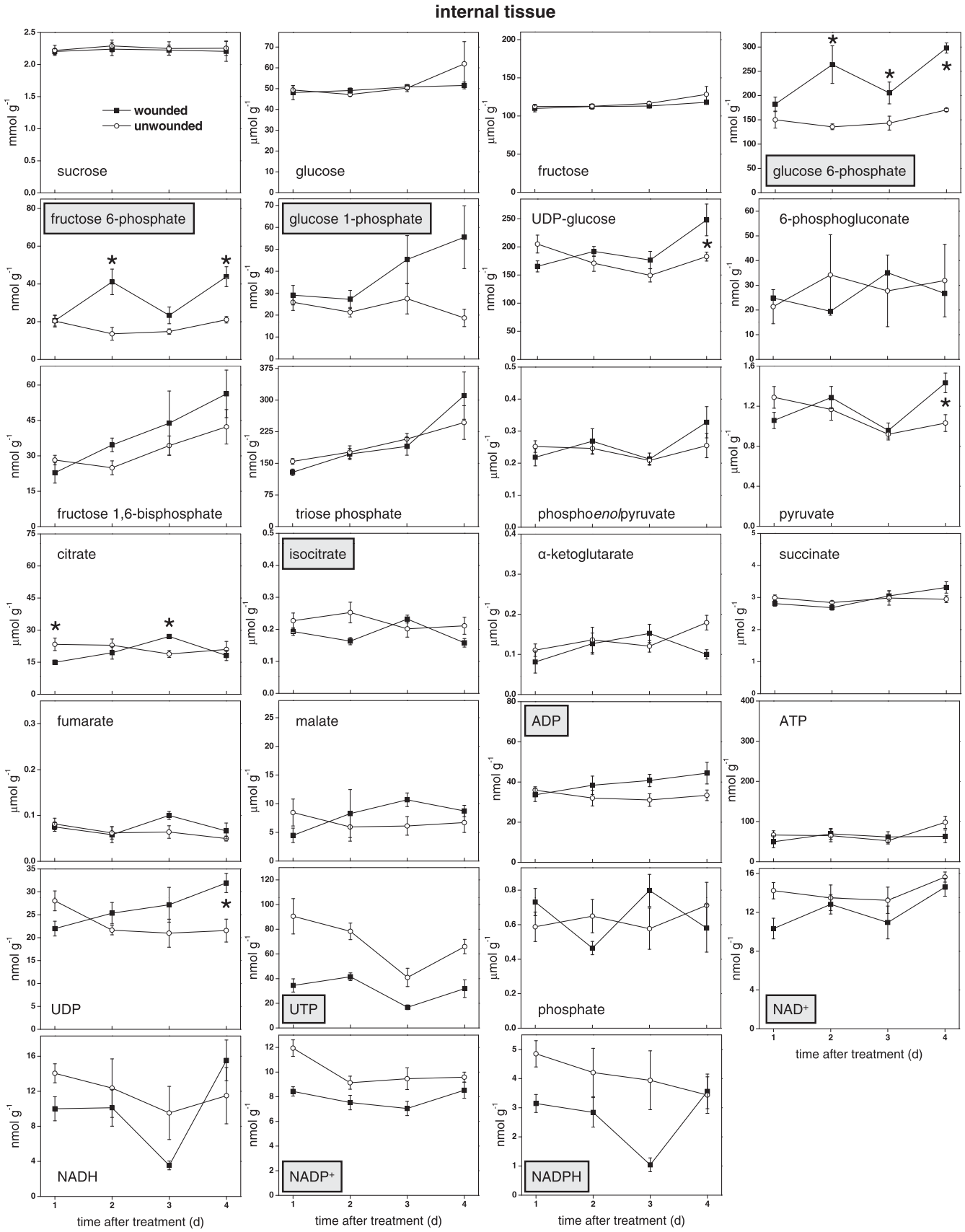

Fig. 5. Concentrations of substrates and intermediates in the respiratory pathway in internal tissues of wounded and unwounded roots during storage at 10 °C for 4 days as a function of time. Internal tissue contained tissue that did not sustain any visible damage in wounded roots and comparable tissue from unwounded roots. Concentrations were determined on a dry weight basis. Metabolites that are statistically different ($\alpha = 0.05$) between wounding treatments are highlighted by a shaded box surrounding the metabolite name. Statistically significant treatment × day interactions are identified with asterisks. Data are the mean ± SE of the mean ($n = 4$).

the TCA cycle were altered after root injury (Fig. 5), indicating systemic effects of injury on primary carbon metabolism. In the internal tissue of roots, wounding was associated with significant elevations in glucose 6-phosphate, fructose 6-phosphate, glucose 1-phosphate and ADP concentrations throughout the duration of the experiment, an increase in citrate concentration exclusively on the 3rd day after injury and increases in UDP-glucose, pyruvate and UDP concentrations exclusively on the fourth day after injury. Wounding was associated with significant reductions in isocitrate, UTP, NAD^+, $NADP^+$ and NADPH concentrations throughout the duration of the experiment, and a reduction in citrate concentration exclusively on the first day after injury. Wounding had no statistically significant effect on the concentrations of sucrose, glucose, fructose, 6-phosphogluconate, fructose 1,6-bisphosphate, triose phosphate, phospho*enol*pyruvate, α-ketoglutarate, succinate, fumarate, malate, ATP, phosphate, or NADH in internal tissues.

The hexose monophosphates, glucose 6-phosphate, fructose 6-phosphate, and glucose 1-phosphate increased in the non-wounded tissue of wounded roots relative to similar tissue in unwounded roots. G6P, F6P and G1P concentrations increased an average of 58%, 85% and 68%, respectively, in wounded roots. Despite some minor differences in their rate and magnitude of elevation, the general increase in the three hexose monophosphates suggests that phosphoglucose isomerase and phosphoglucomutase, the two enzymes responsible for interconversion of the hexose monophosphates, were probably not limiting in internal tissues of wounded roots. This contrasts with the lack of interconversion of these compounds in wounded tissue (Fig. 4). While it was hypothesized that phosphoglucose isomerase and phosphoglucomutase activities limited interconversion of the hexose monophosphates in wounded tissue, flux through these reactions was likely to be significantly greater in wounded tissue relative to the non-wounded tissue of wounded roots since respiration was nearly twofold greater in wounded tissue than in internal tissue (Fig. 3). The overall increase in hexose monophosphates in non-wounded tissue of wounded roots indicates that hexose monophosphate synthesis exceeded usage. This suggests that carbon flux through hexokinase and fructokinase-catalyzed reactions exceeded carbon flux through the reactions catalyzed by phosphofructokinase, glucose 6-phosphate dehydrogenase and UDP-glucose pyrophosphorylase, the enzymes that provide substrate for downstream glycolytic enzymes, the oxidative pentose phosphate pathway and cell wall biosynthesis.

Glycolytic intermediates downstream of the hexose monophosphates were not altered by wounding except for an increase in pyruvate concentration occurring 4 days after injury. Elevated hexose monophosphate concentrations and unaltered concentrations of downstream glycolytic intermediates including fructose 1,6-bisphosphate, triose phosphate and phospho*enol*pyruvate suggest a restriction in the glycolytic pathway at phosphofructokinase in the unwounded tissues of wounded roots. Metabolite concentrations in wounded root tissue also suggested a restriction in glycolytic metabolism due to limited PFK activity (Fig. 4).

Citrate and isocitrate concentrations were significantly altered in the non-wounded tissue of wounded roots relative to similar tissue from unwounded roots. CIT concentrations in the internal tissue of wounded roots were reduced 36% on the first day after injury and elevated 43% on the third day after injury. ISOC concentrations were reduced an average of 16% during the 4-day duration of the experiment. Reductions in CIT and ISOC concentrations were also observed in wounded tissue, although the magnitude of concentration changes was much greater in wounded tissue than in the non-wounded tissue of wounded roots (Fig. 4). In wounded tissue, it was hypothesized that reductions in CIT and ISOC may have been due to engagement of the glyoxylate cycle, which would allow carbon from damaged membranes to be reassimilated in metabolism. In internal tissues, engagement of the glyoxylate cycle is possible, although a function for its engagement is not obvious. Despite concentration changes in CIT and ISOC, the downstream TCA cycle intermediates, α-ketoglutarate, succinate, fumarate and malate were unaltered in concentration in internal tissue of wounded roots.

Wounding altered ADP concentrations in internal tissues. ADP concentrations increased with time after injury and were elevated 33% by the fourth day after injury. This increase in ADP concentration was similar in magnitude to the increase in ADP concentration that occurred in wounded tissue (Fig. 4). ATP concentration in internal tissue, however, was unaffected by wounding, suggesting that ATP synthesis by oxidative phosphorylation kept pace with ATP utilization. This contrasts with the steady reduction of ATP with time after injury that occurred in wounded tissue.

Concentrations of uridine nucleotides were altered in the internal tissues of wounded roots. UTP concentrations dramatically declined 1 day after injury and were reduced an average of 55% in the non-wounded tissue of wounded roots. In contrast, UDP and UDP-glucose concentrations generally increased with time after injury and were significantly elevated in internal tissues of wounded roots 4 days after injury. The cause for the large decline in UTP concentration in the internal tissue of wounded roots is unknown, but was similar to the decrease in UTP concentration that occurred in wounded tissue (Fig. 4). In wounded tissues, changes in UTP concentrations were hypothesized to occur due to callose or cellulose biosynthesis, use of UTP as a substrate in phosphorylation reactions, or interconversion of adenine and uridine nucleotide pools by nucleoside 5′-diphosphate kinase. In the internal tissue of wounded roots, however, it is unlikely that the decline in UTP concentration was due to callose and/or cellulose biosynthesis or equilibration between adenine and uridine nucleotides. In internal tissue, callose and cellulose biosyntheses were expected to be minimal since cells and cell walls in this tissue experienced minimal damage. Equilibration of nucleotides via nucleoside 5′-diphosphate kinase was also unlikely since reductions in UTP concentration were not mirrored by reductions in ATP concentration. Although it is possible that UTP was consumed by a UTP-dependent fructokinase, it is unknown whether this activity is present in sugarbeet.

Concentrations of pyridine nucleotides were reduced in the internal tissues of wounded roots, suggesting that there was a systemic catabolism of these compounds in response to injury. NAD^+, $NADP^+$ and NADPH concentrations were significantly reduced an average of 14%, 21% and 36%, respectively, in internal tissues during the 4-day duration of the experiment. NADH concentrations were also generally reduced, although this reduction was not statistically significant. Reductions in pyridine nucleotide concentrations had no effect on the ratio of NADH:NAD^+ or NAD(P)H:-NAD(P)$^+$ (data not shown). The ratio of NADPH:$NADP^+$, however, was reduced by 20% in internal tissues of wounded roots relative to similar tissue in unwounded roots (data not shown). The systemic effect of wounding on pyridine nucleotide concentrations contrasts with the localized effect of wounding on the concentrations of these compounds. At the wound site, only NAD^+, which was reduced in concentration an average of 59%, was affected by injury (Fig. 4). The general reduction in pyridine nucleotides in internal tissue of wounded roots may occur due to elevated activity of poly(ADP-ribose) polymerase as was hypothesized for the reduction in NAD^+ in the peripheral tissue of wounded roots (Amor et al., 1998; De Block et al., 2005).

2.4. Relationships between wound responses of respiratory pathway substrates, respiratory pathway intermediates, energy charge, redox status and respiration rate

Relationships between the wound responses of respiratory pathway substrates, respiratory pathway intermediates, cellular

energy charge, cellular redox status and respiration rate were determined using correlation and principal component analyses. Cellular energy charge, measured by the ATP:ADP ratio or adenylate charge (AEC; Arrivault et al., 2009), and cellular redox status, measured by the ratio of NADH:NAD^+, NADPH:$NADP^+$ or NAD(P)H:$NAD(P)^+$, were included in these analyses since they have been suggested to regulate glycolytic and TCA cycle reactions, respectively (Day and Lambers, 1983; Riewe et al., 2008; Tcherkez et al., 2009). Respiration rate was also included in the analyses as the final step in the respiratory pathway.

In peripheral tissue, fructose was the only simple sugar whose concentration changes in response to wounding were significantly correlated with those of other metabolites (Table 2). Changes in fructose concentration due to wounding were similar to changes observed for fumarate, UDP, NADH and the ratio of NAD(P)H to $NAD(P)^+$. While correlations may be coincidental, similarities between wound effects on fructose and UDP indicate the possibility that increases in UDP, a substrate for the reversible enzyme sucrose synthase which catalyzes formation of fructose and UDP-glucose from UDP and sucrose, may drive the sucrose synthase reaction toward fructose formation. Fructose concentration changes were also positively correlated with NADH and the ratio of NAD(P)H to $NAD(P)^+$, two indicators of cellular redox status. Although no relationship between fructose metabolism and redox status is known, fructose 6-phosphate, the product of fructokinase reaction and the immediate downstream product of fructose catabolism, was negatively correlated with several indicators of cellular redox status.

Among glycolytic intermediates, only fructose 6-phosphate and glucose 1-phosphate were significantly correlated in their response to wounding. F6P concentration changes were negatively correlated with G1P concentration changes, providing further evidence that hexose monophosphates were not in equilibrium in wounded sugarbeet root tissue. Although ATP, ADP, the ratio of ATP:ADP and adenylate energy charge have been suggested to influence glycolytic flux as substrates, products or effectors of hexokinase, fructokinase, phosphofructokinase, and pyruvate kinase activities (Farrar, 1985), their changes in response to wounding were not significantly correlated with those of any glycolytic intermediate.

No significant correlations were observed between any TCA cycle intermediates, although wound-related changes in citrate and isocitrate concentrations were positively correlated with adenylate energy charge and the ratio of ATP:ADP, respectively, and fumarate concentration changes were positively correlated with several indicators of cellular redox status. The significant correlations between energy charge and citrate and isocitrate concentrations are unrelated to the operation of the TCA cycle, but may be indicative of the engagement of the glyoxylate cycle in wounded root tissue. Adenylates are not involved in the catabolism of citrate and isocitrate via the TCA cycle and activity of the TCA cycle is generally thought to be insensitive to cellular energy charge (Dry and Wiskich, 1982; Møller and Palmer, 1984). Lipid turnover, which generates the acetyl CoA needed for the glyoxylate cycle, however, requires ATP for conversion of fatty acids into their acetyl CoA esters prior to their degradation. A cause for the positive correlation between fumarate concentration changes and NADH and the ratios of NADPH:$NADP^+$ and NAD(P)H:$NAD(P)^+$ is not apparent. Although redox status has been suggested to regulate carbon flux through the TCA cycle by restricting activity of the cycle's NAD-dependent dehydrogenase activities (Møller and Palmer, 1984; Tcherkez et al., 2009), fumarate synthesis or catabolism is not directly affected by these enzymes.

Changes in respiration rate in response to wounding were not correlated to wound-related changes in any respiratory pathway substrate or intermediate, cellular energy charge, or cellular redox status. Similarly, changes in ADP concentration in response to wounding were not correlated to wound-related changes in any respiratory pathway substrate or intermediate, cellular redox

Table 2
Significant relationships in the response of respiratory pathway substrates, respiratory pathway intermediates, cellular energy charge, redox status and respiration rate (RESP) to wounding in peripheral tissues of roots during 4 days storage at 10 °C. Significant relationships were determined by Pearson product moment correlations at $\alpha = 0.05$. Peripheral tissue contained the visibly damaged tissue in wounded roots. Cellular energy charge was measured as the ATP:ADP ratio or adenylate energy charge (AEC). Redox status was measured as the ratios of NADH:NAD^+, NADPH:$NADP^+$, or NAD(P)H:$NAD(P)^+$. Respiration rate was measured as oxygen consumption. Abbreviations used for metabolites are defined in Table 1. – is used to denote the absence of any significant correlations. Correlation coefficients for all comparisons can be found in Supplementary data.

Metabolite	+ Correlations	− Correlations
SUC	–	–
GLU	–	–
FRU	FUM, UDP, NADH, NAD(P)H:NAD(P)	–
G6P	–	–
F6P	–	G1P, FUM, PHOS, NADH, NADPH:NADP; NAD(P)H:NAD(P)
G1P	6PGLU, FUM, PHOS, NAD(P)H:NAD(P)	F6P
UDPG	PYR	–
6PGLU	G1P, FBP	CIT
FBP	6PGLU	–
TRIOP	–	–
PEP	–	SUCC
PYR	UDPG	–
CIT	AEC	6PGLU
ISOC	ATP:ADP	UTP
KETOG	–	–
SUCC	–	PEP
FUM	FRU, G1P, PHOS, NADH, NADPH:NADP, NAD(P)H:NAD(P)	F6P
MAL	NADP	NAD
ADP	–	–
ATP	ATP:ADP	UDP, UTP
UDP	FRU, NADH	ATP
UTP	–	ISOC, ATP, ATP:ADP
PHOS	G1P, FUM, NAD(P)H:NAD(P)	F6P
NAD	–	MAL, NADP
NADH	FRU, FUM, UDP, NAD(P)H:NAD(P)	F6P
NADP	MAL	NAD
NADPH	NADH:NAD; NADPH:NADP	–
RESP	–	–

status or respiration rate, even though ADP concentration has been implicated in control of glycolytic flux and respiration rate in several plant species and organs (Farrar, 1985; Loef et al., 2001).

In internal tissues of wounded roots, significant correlations were observed between wound-associated changes in the concentrations of neutral sugars, glycolytic intermediates, indicators of cellular redox status and respiration rate (Table 3). Among the neutral sugars, a positive correlation was found for wound-associated changes in glucose and fructose concentrations. The positive correlation between glucose and fructose concentrations is suggestive of wound-induced degradation of sucrose in internal tissues by invertase, an enzyme that hydrolyzes sucrose to glucose and fructose. However, while invertase-catalyzed degradation of sucrose in sugarbeet root tissue is possible and has been proposed (Rosenkranz et al., 2001), sucrose catabolism in sugarbeet root tissue, as in most heterotrophic organs, is thought to be catalyzed primarily by sucrose synthase, an enzyme that catalyzes the reaction of sucrose with UDP to form UDP-glucose and fructose (Sung et al., 1989; Klotz and Finger, 2004). Alternatively, the correlation between glucose and fructose could arise from a coordinated catabolism of these compounds by hexokinase and fructokinase, respectively, and is consistent with the positive correlations of glucose and fructose concentrations with the ratio of ATP to ADP. While it is unknown whether coordinated changes in hexokinase and fructokinase are responsible for the positive correlation between glucose and fructose in internal root tissues, it is known that both enzymes are induced in sugarbeet roots by wounding (Klotz et al., 2006).

Several glycolytic intermediates were positively correlated in their responses to wounding in internal root tissue. Glucose 6-phosphate concentrations were positively correlated with fructose 6-phosphate concentrations, suggesting that their synthesis by hexokinase and fructokinase was coordinated or that they were readily interconverted by phosphoglucose isomerase. Wound-related concentrations changes in G6P and F6P were also positively correlated with UTP concentration changes, suggesting the possible operation of a UTP-dependent fructokinase in sugarbeet root tissue. Positive correlations were also found between wound-related concentration changes of glycolytic intermediates downstream of fructose 1,6-bisphosphate, i.e., triose phosphate, phospho*enol*pyruvate and pyruvate. The correlation between TRIOP and PEP suggests that the reversible enzymes that catalyze the interconversions between these two glycolytic intermediates were at equilibrium, while their correlation with pyruvate suggests a coordination or dependence of pyruvate formation and catabolism by the irreversible enzymes pyruvate kinase and pyruvate dehydrogenase on the concentrations of glycolytic precursors. Similar to peripheral tissues (Table 2), ATP, ADP, the ATP:ADP ratio and adenylate energy charge were unrelated to concentration changes of any glycolytic intermediates.

No significant correlations were observed between any TCA cycle intermediates, although wound-related concentration changes in α-ketoglutarate were negatively correlated with several indicators of cellular redox status and respiration rate. The negative correlations between α-ketoglutarate and the ratios of NADH:NAD^+, NADPH:$NADP^+$, and NAD(P)H:$NAD(P)^+$ may be due to control of KETOG formation by cellular redox status since ratios of NADH:NAD^+ and NADPH:$NADP^+$ have been implicated in the regulation of isocitrate dehydrogenase, the TCA cycle enzyme responsible for KETOG formation (Igamberdiev and Gardeström, 2003). While the significance of the negative relationship between KETOG concentration changes and respiration rate in the internal tissues of sugarbeet root is unknown, KETOG catabolism by α-ketoglutarate dehydrogenase has been suggested to have a role in limiting respiration in potato tubers (Araújo et al., 2008).

Wound-related changes in respiration rate in internal root tissue were positively correlated with all indicators of cellular redox status. Respiration rate was positively correlated with

Table 3
Significant relationships in the response of respiratory pathway substrates, respiratory pathway intermediates, cellular energy charge, redox status and respiration rate (RESP) to wounding in internal tissues of roots during 4 days storage at 10 °C. Significant relationships were determined by Pearson product moment correlations at $\alpha = 0.05$. Internal tissue sustained no visible damage in wounded roots. Cellular energy charge was measured as the ATP:ADP ratio or adenylate energy charge (AEC). Redox status was measured as the ratios of NADH:NAD^+, NADPH:$NADP^+$, or NAD(P)H:$NAD(P)^+$. Respiration rate was measured as oxygen consumption. Abbreviations used for metabolites are defined in Table 1. – is used to denote the absence of any significant correlations. Correlation coefficients for all comparisons can be found in Supplementary data.

Metabolite	+ Correlations	− Correlations
SUC	–	G6P, UTP, NAD
GLU	FRU, ATP:ADP	–
FRU	GLU, ATP:ADP	–
G6P	F6P, UTP, NAD	SUC
F6P	G6P, UTP	–
G1P	SUCC	–
UDPG	ADP, UDP	–
6PGLU	PHOS	UTP
FBP	–	–
TRIOP	PEP, PYR	–
PEP	TRIOP, PYR, NADP	–
PYR	TRIOP, PEP, NADP	–
CIT	–	–
ISOC	–	–
KETOG	ATP	NADH:NAD, NADPH:NADP, NAD(P)H:NAD(P), RESP
SUCC	G1P	–
FUM	–	–
MAL	–	–
ADP	UDPG, UDP	–
ATP	KETOG	–
UDP	UDPG, ADP	–
UTP	G6P, F6P, NAD	SUC, 6PGLU
PHOS	6PGLU	–
NAD	G6P, UTP	SUC
NADH	NADPH, NADH:NAD, NAD(P)H:NAD(P), RESP	–
NADP	PEP, PYR	–
NADPH	NADH, NADH:NAD, NADPH:NADP, NAD(P)H:NAD(P), RESP	–
RESP	NADH, NADPH, NADH:NAD, NADPH:NADP, NAD(P)H:NAD(P)	KETOG

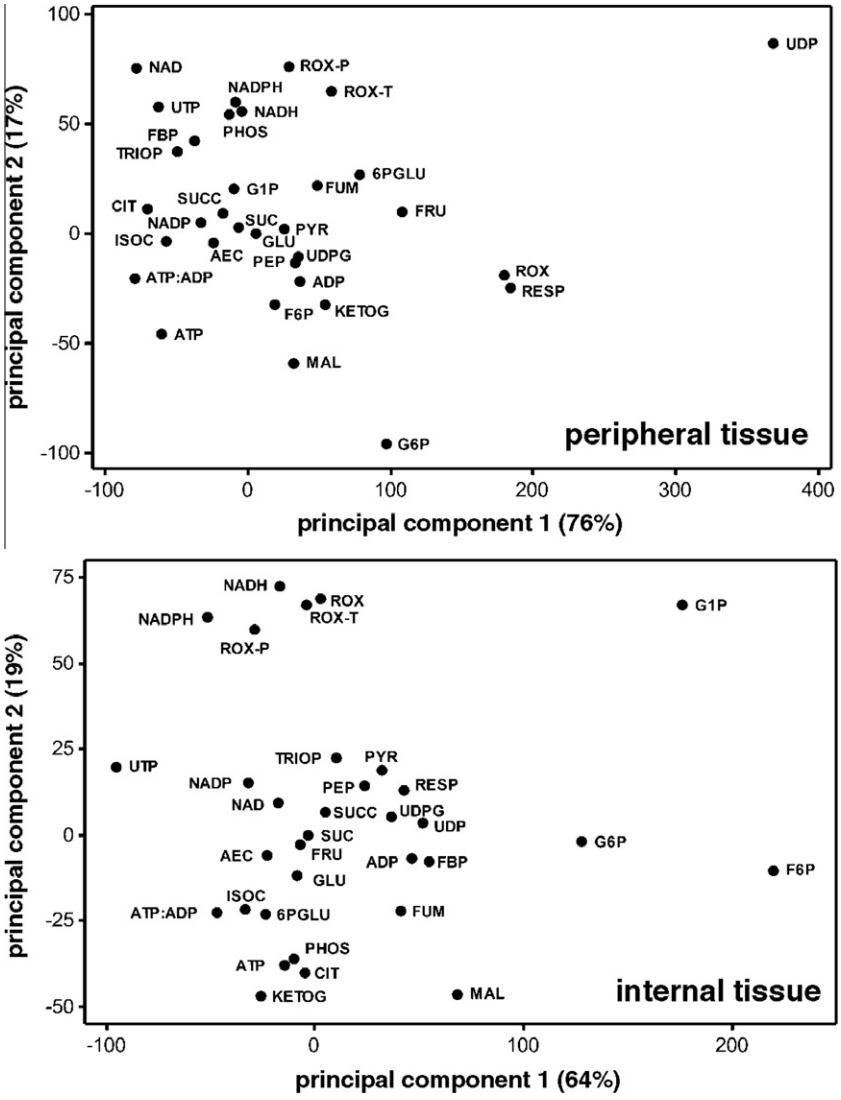

Fig. 6. Score plots from principal component analyses of the wound response of respiratory pathway substrates, respiratory pathway intermediates, energy charge, redox status and respiration rate in peripheral and internal tissues of roots during 4 days storage at 10 °C. Peripheral tissue contained the visibly damaged tissue from wounded roots and comparable tissue from unwounded roots; internal tissue contained tissue that did not sustain any visible damage. The proportion of the variance described by each principal component is provided in parentheses in each axis title. Abbreviations used to describe metabolites are presented in Table 1. Additional abbreviations used: AEC, adenylate energy charge; RESP, respiration; ROX, NADH:NAD^+ ratio; ROX-P, NADPH:$NADP^+$ ratio; ROX-T, NAD(P)H:$NAD(P)^+$ ratio. Loading plots can be found in Supplementary data.

wound-related changes in the concentrations of NADH and NADPH and the ratios of NADH:NAD^+, NADPH:$NADP^+$, and NAD(P)H:-$NAD(P)^+$. Control of respiration rate in sugarbeet root by redox status has not been previously suggested. However, a positive association between sugarbeet root mitochondrial respiration rate and NAD^+ concentrations has been demonstrated which led to the suggestion that sugarbeet root respiration rate is limited by NAD^+ availability (Shugaev, 2001).

Principal component analysis (PCA) was also used to elucidate similarities in the wound response of respiratory pathway substrates, respiratory pathway intermediates, cellular energy charge, cellular redox status and respiration rate (Fig. 6). In peripheral tissues, PCA confirmed the similarity in the response of fructose 1,6-bisphosphate and triose phosphate concentrations to wounding that suggested that these compounds were in equilibrium in wounded tissue (Fig. 4). PCA also confirmed the dissimilarity in the responses of glucose 6-phosphate, fructose 6-phosphate and glucose 1-phosphate concentrations to wounding (Fig. 4, Table 2), providing additional evidence that equilibration of these metabolites did not occur in wounded tissue. New relationships were also established by PCA and included similarities in the effect of wounding on concentration changes of phospho*enol*pyruvate and pyruvate, concentration changes of citrate and isocitrate, and respiration rate and the ratio of NADH:NAD^+. The similarity in concentration changes of PEP and PYR, the substrate and product of the irreversible pyruvate kinase reaction, suggests a dependence of PYR concentration on PEP concentration in wounded tissue. In the internal, non-wounded tissues, a similar effect of wounding on PEP and PYR concentrations was also found by correlation analysis (Table 3). The significance of the similarity in the effect of wounding on respiration rate and the ratio of NADH:NAD^+ is unknown, although respiration rate and redox status were found to be positively correlated in internal tissues (Table 3) and a relationship between respiration rate and NAD^+ concentration has been reported previously (Shugaev, 2001).

In internal tissues, PCA confirmed previously noted associations between glucose and fructose, between triose phosphate, phospho*enol*pyruvate and pyruvate, and between UDP and UDP-glucose. Although correlation analysis indicated a positive association between respiration rate and indicators of redox status

(Table 3), no such similarity was indicated by PCA. Rather, in the non-wounded tissues of wounded roots, PCA indicated that wound-related changes in respiration rate were most similar to wound-related changes in pyruvate and UDP-glucose concentrations.

3. Conclusions

Characterization of wound-related changes in respiration rate and the concentrations of metabolites that are substrates, intermediates or cofactors in the respiratory pathway provided clues to the changes in primary carbon metabolism that occur in sugarbeet roots in response to injury. In wounded root, respiration and metabolite concentration changes occurred in wounded tissue and in tissue internal to the wound site, indicating that localized and systemic changes in primary carbon metabolism occurred in response to injury. Concentration changes observed for fructose, fructose 6-phosphate, glucose 6-phosphate, glucose 1-phosphate and fructose 1,6-bisphosphate in wounded tissue suggested that the early glycolytic enzymes, fructokinase, phosphofructokinase, phosphoglucose isomerase and phosphoglucomutase, were likely to be limiting. In non-wounded tissue of wounded roots, where the demand for glycolytic intermediates was presumably lower, concentration changes for these same metabolites suggested that only phosphofructokinase was limiting. In wounded tissue, reductions in the concentration of early glycolytic and early TCA cycle intermediates but unaltered concentrations of downstream metabolites suggested that carbon compounds were likely to be infused into downstream glycolytic reactions and the TCA cycle via metabolic bypasses such as a phospho*enol*pyruvate carboxykinase-catalyzed production of phospho*enol*pyruvate, the glyoxylate cycle, or amino acid catabolism. While entry of carbon compounds into the respiratory pathway via alternative metabolic pathways has not been proven, it provides a ready explanation of how respiration rate in wounded tissue was able to increase twofold with little or no reduction in the immediate substrates of respiration despite an apparent restriction in carbon flux by early glycolytic enzymes. In the non-wounded tissue of wounded roots, reductions in respiratory pathway intermediates were limited to a transient decline in citrate and a small decrease in isocitrate concentration. This suggests that glycolysis and the TCA cycle were generally capable of providing the substrates needed to support the small elevation in respiration rate that occurred in internal tissues. Changes in ATP concentrations indicate that oxidative phosphorylation failed to keep pace with ATP utilization in wounded tissue. However, no evidence was found that alterations in ADP concentration, ATP concentration, the ratio of ATP:ADP or adenylate energy charge had any effect on carbon flow through glycolysis or respiration in either the wounded or unwounded tissues of wounded roots. Interestingly, a positive association between wound-induced respiration and alterations in cellular redox status was indicated by principal component analysis in wounded tissue and by correlation analysis in the non-wounded tissue of wounded roots. The significance of this observation is unknown but suggests that the possible regulation of respiration rate in sugarbeet root by redox status deserves further study.

4. Experimental

4.1. Plant materials and postharvest treatments

Sugarbeet (*B. vulgaris* L.) variety, Beta 6225 (Betaseed Inc., Shakopee, MN, USA), was grown in a greenhouse in 15 L pots for 17 weeks with 16 h days and 8 h nights after which roots were harvested, all leaves and vegetative buds were removed, and roots were gently washed. Roots were randomly assigned to two groups. One group was severely wounded by tumbling in a pilot-scale beet washer (Hallbeck, 1982) for 30 min, causing extensive abrasion of the root surface and severe bruising. The second group was unwounded and served as a control. After treatment, all roots were stored for 4 days at 10 °C and 90% relative humidity. No symptoms of microbial infection were evident on any roots during the experiment. Tissue samples were collected daily from the widest portion of the roots during 4 days storage and separated into peripheral tissue comprised of tissue immediately below the epidermis to a depth of 1 cm and internal tissue comprised of tissue $\geqslant$2 cm below the epidermis but external to the central vascular cylinder. Peripheral tissue contained the visibly damaged tissue from wounded roots and comparable tissue from unwounded roots; internal tissue contained tissue that did not sustain any visible damage. Tissue samples were flash frozen in liq. N_2, lyophilized, ground to a fine powder, and stored at −80 °C until analysis. Experiments were conducted with four replications with two roots per replicate.

4.2. Respiration rate determinations

Respiration rate of whole roots was determined as CO_2 efflux by infrared CO_2 analysis using a LICOR 6400 gas analyzer (Lincoln, NE, USA) modified for use with a 7 L sample chamber (Haagenson et al., 2006). Respiration rate of tissue samples was measured as O_2 consumption at 25 °C using a Clark-type oxygen electrode (Hansatech Oxytherm, Norfolk, England) as previously described (Klotz et al., 2008). Oxygen consumption was measured after 1, 2, 3 and 4 days of storage from fresh peripheral and internal tissues. Respiration rates for whole roots and tissue sections were determined independently with a second set of wounded and unwounded roots with similar results.

4.3. Metabolite extractions

For determination of carbohydrate, organic acid, and nucleotide concentrations, lyophilized tissue (50 mg) was extracted in MeOH–H_2O (1 mL, 4:1, v/v) according to the method of De Bruijn et al. (1999). Samples were boiled for 15 min at 75 °C, solvent was removed by evaporation, and extracts were redissolved in H_2O (1 mL). Undissolved material was removed by centrifugation at 16,000*g* for 10 min. For determination of pyridine nucleotide concentrations, lyophilized tissue was extracted using the method of Matsumura and Miyachi (1980). NAD^+ and $NADP^+$ were extracted from tissue with 0.1 N HCl at 100 °C for 2 min, after which the solution was neutralized with 0.1 N NaOH and centrifuged at 14,000*g* for 10 min at 4 °C to remove insoluble material. For NADH and NADPH determinations, tissue was extracted with 0.1 N NaOH at 100 °C for 2 min, neutralized with 0.1 N HCl and centrifuged at 14,000*g* for 10 min at 4 °C.

4.4. Metabolite quantifications by HPLC

Sucrose, glucose, and fructose concentrations were determined after 90-fold dilution of extracts, injection onto a 250 × 4 mm CarboPac PA 1 column (Dionex, Sunnyvale, CA, USA) equipped with a 50 × 4 mm PA 1 guard column, elution with 62 mM NaOH at 1 mL min^{-1}, and detection with an electrochemical detector (Dionex ED50) operating in pulsed amperometric mode using the manufacturer's recommended settings for carbohydrate analysis. 6-Phosphogluconate was determined as above except using a two-component gradient composed of (A) 100 mM NaOH and (B) 100 mM NaOH and 1 M NaOAc, where component B increased from 10% to 20% between 0 and 20 min, increased to 50% between 20 and 30 min and remained at 50% for an additional 5 min. Phospho*enol*pyruvate, pyruvate, citrate, α-ketoglutarate, fumarate,

malate, AMP, ADP and ATP concentrations were determined after 10-fold dilution of extracts, injection onto a 250 × 4.6 mm Prevail organic acid column (Grace Davison Discovery Science, Deerfield, IL, USA), elution with 25 mM KH_2PO_4 (pH 2.5) at 1 mL min^{-1}, and spectroscopic detection at 210 nm. UDP, UTP and UDP-glucose concentrations were determined by spectroscopic detection at 254 nm after passage of extracts over a 250 × 4.6 mm Partisil SAX anion-exchange column (Alltech Associates, Deerfield, IL, USA), eluted at 1 mL min^{-1} with a gradient comprised of (A) 10 mM $NH_4H_2PO_4$ (pH 2.8) and (B) 750 mM $NH_4H_2PO_4$ (pH 3.7), in which component B was held at 7.5% for 12 min, increased to 90% over 3 min, increased to 100% over 10 min and held at 100% for an additional 7 min (Geigenberger et al., 1997). External standards and calibration curves were used to identify and quantify metabolites.

4.5. Metabolite quantifications by spectroscopic assays

Glucose 6-phosphate, fructose 6-phosphate, and glucose 1-phosphate concentrations were determined from the change in absorbance of solutions containing 100 mM Tris–HCl (pH 8.1), 5 mM $MgCl_2$, 0.25 mM $NADP^+$ and tissue extract in a total volume of 175 μL after sequential additions of 0.2 U glucose-6-phosphate dehydrogenase (G6PDH), 0.2 U phosphoglucose isomerase, and 0.06 U phosphoglucomutase (Stitt et al., 1989). Reactions were carried out at 25 °C and absorbance measured at 340 nm with a SpectraMAX Plus microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA). Triose phosphate concentrations, due to the combined concentrations of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, and fructose 1,6-bisphosphate concentrations were determined as described above except $NADP^+$ was replaced by 150 μM NADH and absorbance changes determined after sequential additions of 0.25 U glycerophosphate dehydrogenase, 0.6 U triose phosphate isomerase, and 0.05 U aldolase (Bergmeyer, 1993). Isocitrate concentrations were determined from the change in absorbance at 340 nm of solutions containing 50 mM Tris–HCl (pH 8.1), 0.3 mM $NADP^+$, 0.1 mM $MnCl_2$, 0.4 U isocitrate dehydrogenase and tissue extract in a total volume of 175 μL after incubation at 25 °C (Passonneau and Lowry, 1993). Succinate concentrations were determined using a succinic acid assay kit purchased from Megazyme (Wicklow, Ireland). NAD^+ and NADH concentrations were determined by the change in absorbance at 570 nm after a 30 min, 37 °C incubation of a solution containing 100 mM bicine-NaOH buffer (pH 8.0), 4 mM EDTA, 0.42 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 1.7 mM phenazine ethosulfate (PES), 500 mM ethanol, 2 U alcohol dehydrogenase (ADH), and extract in a total volume of 200 μL (Matsumura and Miyachi, 1980). Quantification of $NADP^+$ and NADPH was similar to that of NAD^+ and NADH except EtOH was replaced with 2.5 mM G6P and ADH was replaced with 0.14 U glucose-6-phosphate dehydrogenase (Matsumura and Miyachi, 1980). Inorganic phosphate concentrations were determined by the change in absorbance at 630 nm after complexation with an ammonium molybdate solution and reduction with ascorbic acid as previously described (Drueckes et al., 1995). For assays in which NAD^+ was reduced or NADH was oxidized, metabolite concentrations were determined using the extinction coefficient for NADH. For other assays, metabolites were quantified using calibration curves generated with appropriate standards.

4.6. Mathematical and statistical analyses

Adenylate energy charge (AEC; Arrivault et al., 2009) was calculated by the equation, ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]). $NAD(P)^+$ concentrations were determined by addition of NAD^+ and $NADP^+$ concentrations; NAD(P)H concentrations were determined by addition of NADH and NADPH concentrations. Two-way analysis of variance, combined with Tukey's range test, was used to determine significant differences between treatments and days where $\alpha = 0.05$. Principal component analyses (PCA) were performed using Minitab Statistical Software (ver. 16, State College, PA, USA). Analyses were calculated using a covariance matrix with the change in concentration between wounded and control roots used as the input data. Pearson product moment correlation coefficients were determined using Minitab Statistical Software with significance defined as $P \leqslant 0.05$.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.12.016.

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